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THE BINDING OF JUVENILE HORMONE TO LARVAL EPIDERMIS: INFLUENCE OF CARRIER PROTEIN FROM THE HEMOLYMPH OF *PLODIA INTERPUNCTELLA*

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INTRODUCTION

The role of juvenile hormone (JH) in growth and development of insects has been discussed in several recent reviews (Menn and Beroza, 1972; Burdette, 1974; Sláma *et al.*, 1974). The hormone is secreted by the corpora allata and is transported by the hemolymph to target tissues. Lipoproteins and low molecular weight proteins in the hemolymph have been implicated as carriers for JH (Trautmann, 1972; Whitmore and Gilbert, 1972; Emmerich and Hartmann, 1973; Kramer *et al.*, 1974a; Ferkovich *et al.*, 1975). The low molecular weight proteins which bind JH and have been isolated from both *Manduca sexta* (Sanburg *et al.*, 1975a,b) and *Plodia interpunctella** protect JH against nonspecific esterases in fourth- and early fifth-instar larvae. JH specific esterases that degrade the protein-bound JH also appear in the hemolymph during the late fifth instar and may provide a mechanism for removing JH from the hemolymph to permit metamorphosis.

In addition to its protective role in the hemolymph, JH carrier protein may be involved in the interaction of JH with receptor sites within the cells of target tissues. Although it has been postulated that JH acts at the nuclear (genetic) (Sláma *et al.*, 1974) or cytoplasmic level (Firstenberg and Silhacek, 1973), the primary site(s) of action has not been identified.

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Sláma et al. (1974) have hypothesized that JH binds to receptors in target tissues that mediate characteristic developmental responses in insects and "recognize" the hormone on the basis of its size, shape, and physiochemical properties. Only two studies directed toward the isolation of receptors for JH have been reported. Schmialek (1973) used a radiolabeled analogue of JH to isolate a Triton X-100 solubilized ribonucleoprotein receptor from pupal epidermis of Tenebrio molitor. Also, Ferkovich et al. (1974) examined the binding of JH to subcellular fractions of whole larvae of P. interpunctella and showed that the hormone was primarily bound by protein in fractions containing membrane fragments and RNA; however, binding by degradative enzymes was not ruled out.

We have examined the effects of carrier protein fraction (CPF) on JH binding to epidermal homogenates from larvae of the Indian meal moth, P. interpunctella. We consider what role carrier protein may play in protecting JH from degradation by subcellular fractions of epidermis, and whether CPF is important in recognition of JH by subcellular binding sites.

MATERIALS AND METHODS

The carrier protein fraction (CPF) was isolated from the hemolymph of mid-fifth-instar larvae (12 mg/larva) by gel permeation chromatography as described by Ferkovich et al. (1975). The JH binding in the fractions was measured by separating free JH from bound JH by charcoal absorption (Sanburg et al., 1975a). Esterases were monitored by measuring the 1-napthylacetate hydrolytic activity (Sanburg et al., 1975a) or by measuring metabolites of [^{3}H]-JH by thin-layer chromatography (Slade and Zibitt, 1972). A Sephadex G-100 column (1.5 cm X 90 cm long column at a 27 ml/hr flow rate) separated the majority of the carrier protein from the JH esterases.

Epidermal homogenates were prepared from mid-fifth instar larvae (12 mg/larva) in a Teflon-pestle homogenizer (clearance 0.15-0.22 mm; 10 full strokes at 2500 rpm). The CPF or bovine serum albumin (BSA) (less than 0.005% fatty acids, Sigma) was incubated with [^{3}H]-JH (Hyalophora cecropia C_{18} JH [7-ethyl-1,2-^{3}H(N)] 14.1 Ci/mM; New England Nuclear Corp.) for 15 min at room temperature before incubating with the homogenized epidermis for 30 min. The homogenates were then fractionated according to the scheme presented in Fig. 1.

The sucrose density gradient fractions were not homogeneous. However, the predominant organelles contained in each fraction were: F_1 and F_2, membrane vesicles; F_3, fragments of rough ER,

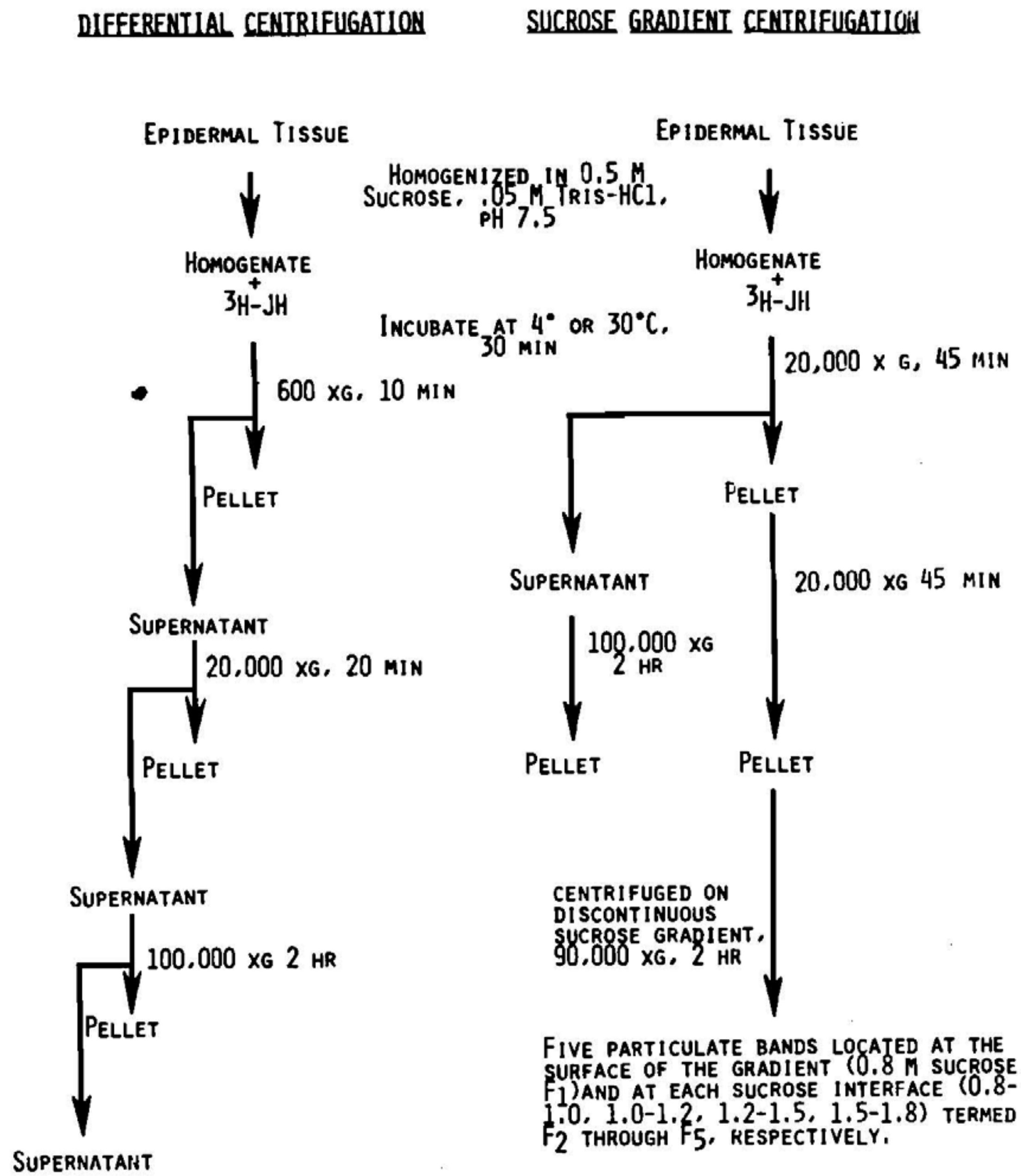

Fig. 1. Procedure for the centrifugation methods used for subcellular fractionation of epidermal tissue incubated with [^{3}H]-JH.

small mitochondria; F_4 rough ER, large mitochondria, lysosomes; F_5, nuclear material, membrane fragments (Ferkovich et al., 1975). To facilitate discussion of JH binding in the particulate fractions, the data obtained on each was combined and is reported as follows: MV, membrane vesicles (F_1 + F_2), M, mitochondrial (F_3 + F_4); and N, nuclear (F_5). The 20,000 X g supernatant is referred to as

M + S, the microsomal + supernatant fraction.

Specific binding in the subcellular fractions was determined according to King and Mainwaring (1974). Homogenized epidermis and [^{3}H]-JH were incubated at 4°C for 15 min. An unlabeled mixture of H. cecropia JH isomers (Hoffmann-LaRoche) was then added to half of the homogenate and both portions were incubated at the appropriate temperature for an additional 15 min.

The protein content of the fractions was determined by a modification of the Lowry method described by Schacterle et al. (1973); and the radioactivity was determined by scintillation counting (Ferkovich et al., 1975).

In certain experiments the epidermal homogenates were incubated with RNase, 500 µg/ml, or DNase (Worthington Biochem. Corp.), 500 µg/ml + 0.06% $MgSO_4$ at 37°C for 30 min prior to incubation with radioactive JH.

RESULTS

Influence of CPF on Binding and Degradation of JH

The radioactivity/µg protein and the percentage of unmetabolized [^{3}H]-JH in fractions obtained from homogenates of epidermis incubated with and without the CPF is shown in Fig. 2 (Ferkovich and Rutter, 1975b). The relative order of radioactivity/µg protein was nuclear (N) < mitochondrial (M) < membrane vesicles (MV) < microsomal + supernatant fractions (M + S). The majority of the label recovered (>94%) remained in the 20,000 X g supernatant that contained the microsomes and cytosol proteins. With the CPF [^{3}H]-JH binding was diminished in all the particulate fractions, probably because of the solubilizing effect of CPF on JH in the supernatant.

In the nuclear and mitochondrial fractions there was more degradation of [^{3}H]-JH in the presence of the CPF; whereas, in the membrane vesicle and microsomal + supernatant fractions the hormone was protected (Table 1). Analyses of the [^{3}H]-JH bound in the nuclear and mitochondrial fractions indicated that the addition of CPF increased the percentage of all the metabolites (Table 2). The diol acid metabolite was highest in the membrane vesicle and microsomal + supernatant fractions and CPF had the most striking protective effect in these fractions.

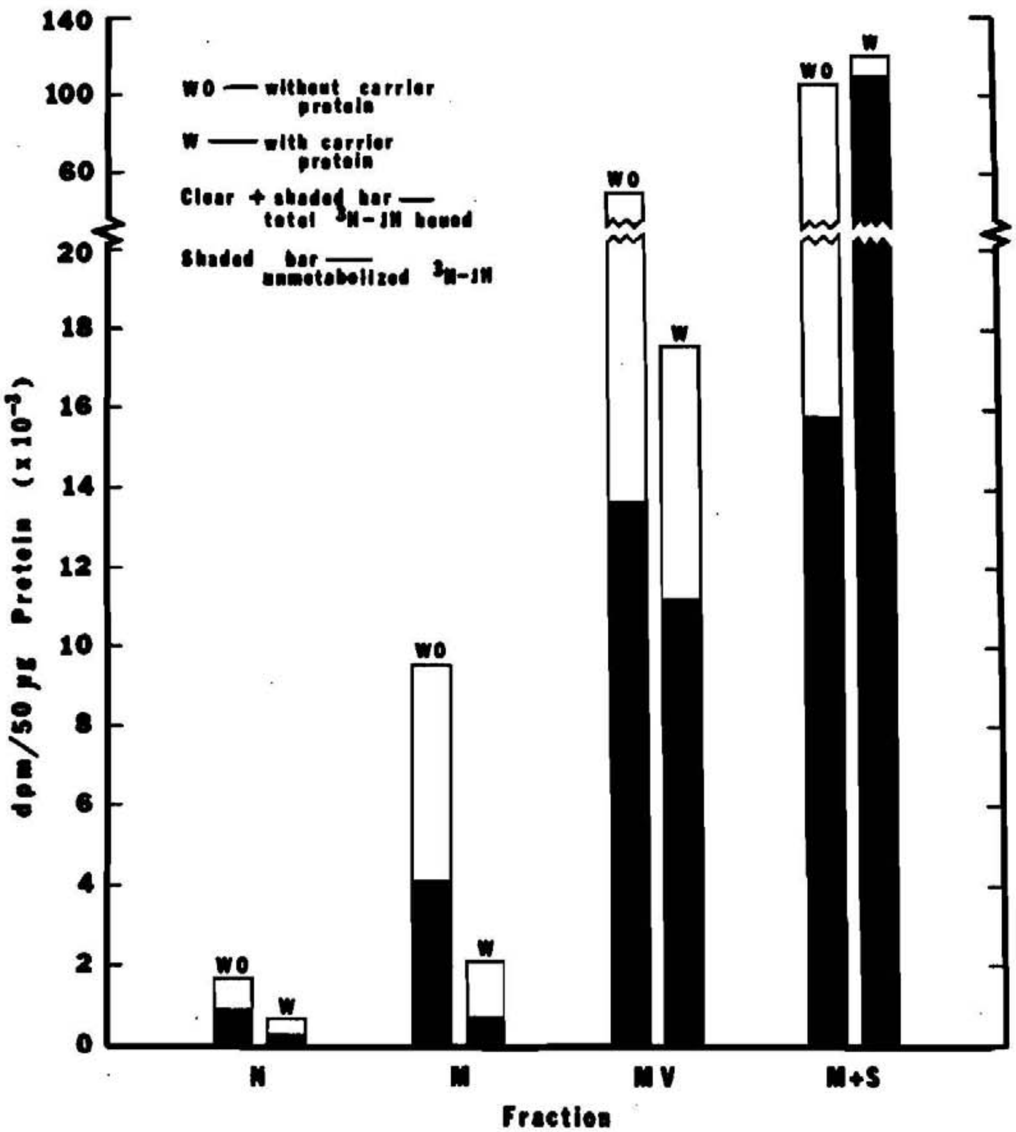

Fig. 2. Subcellular distribution of bound radioactivity after incubation of homogenized epidermis with and without the CPF. Epidermal homogenates (0.12 g wet wt/ml; 150 µg protein 0.1 ml) were incubated with [^{3}H]-JH (8 x 10^{-8}M) with and without the CPF 400 µg/ml at 4°C for 30 min. The fractions were obtained by sucrose gradient centrifugation of the 20,000 X g pellet (Ferkovich and Rutter, 1975b).

Comparisons of CPF and BSA on Binding and Degradation of JH

The binding of JH by BSA has been demonstrated by using UV difference spectroscopy (Ferkovich et al., 1974) and more recently,

TABLE 1. PERCENTAGE OF BOUND [^{3}H]-JH METABOLIZED IN THE SUBCELLULAR FRACTIONS OF HOMOGENIZED EPIDERMIS INCUBATED WITH [^{3}H]-JH IN THE PRESENCE OF CPF.[a]

	% Metabolized JH	
Subcellular fraction	Without CPF	With CPF
Nuclear	44	63
Mitochondrial	56	66
Membrane vesicles	75	35
Microsomal + supernatant	85	8

[a] Epidermal homogenates (0.12 g wet wt/ml; 150 μg protein/0.1 ml) were incubated with [^{3}H]-JH (8×10^{-8} M) plus 400 μg/ml of CPF at 4°C for 30 min.

fluorescence spectroscopy.* We also compared the relative effectiveness of BSA and CPF on binding and degradation of JH in subcellular fractions obtained by differential centrifugation of epidermal homogenates.

In the presence of BSA or CPF the nuclear and microsomal + supernatant fractions contained about 4×10^{10} molecules of JH/μg of protein; the mitochondrial fraction contained about 10^{10} molecules of JH/μg of protein. In the presence of BSA (nuclear and mitochondrial) fractions of epidermal homogenates broke down 49% of the JH compared with 90% by the microsomal + supernatant fractions during a 30 min incubation at 4°C. Substitution of CPF for BSA resulted in a 65% (nuclear and mitochondrial) to 10% (microsomal + supernatant) breakdown of JH during a similar incubation. These data indicate that binding of JH to protein does not in itself afford protection of the JH molecule in the microsomal + supernatant fraction, but that other properties of the CPF confer the protective effect.

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TABLE 2. PERCENTAGE OF JH DEGRADED IN SUBCELLULAR FRACTIONS OF HOMOGENIZED EPIDERMIS WITH AND WITHOUT CPF.

	% JH or metabolite without CPF				% JH or metabolite with CPF			
Fraction	JH	Diol ester	Epoxy acid	Diol acid	JH	Diol ester	Epoxy acid	Diol acid
Nuclear	61.2	16.1	10.8	11.8	42.0	19.4	15.9	22.7
Mitochondrial	50.0	11.9	15.3	22.7	41.2	18.2	15.8	24.9
Membrane vesicles	29.7	12.9	15.7	41.7	69.1	5.5	5.2	20.0
Microsomal + supernatant	15.2	5.1	4.0	75.8	92.9	-	-	7.1

Determination of High-Affinity Binding

To further characterize the nature of the binding in the particulate fractions, homogenates labeled with [^{3}H]-JH were treated with a 1000-fold excess of unlabeled JH. In essence, JH bound to low-affinity sites should be displaced by the unlabeled JH whereas JH bound to high-affinity sites should not be displaced (King and Mainwaring, 1974). Interpretation of the data summarized in Table 3 was complicated by the breakdown of JH simultaneous with its binding to the subcellular components in the homogenate. The excess unlabeled JH decreased the radioactivity in the membrane vesicles fraction indicating low-affinity binding. However, the expected increase in radiolabel in the supernatant did not occur; rather, radiolabel increased in the nuclear, mitochondrial, and microsomal fractions. Our interpretation of these data is that rapid binding of labeled JH to both low- and high-affinity sites occurred initially, but that the amounts of JH bound decreased during the remainder of the 30 min incubation due to breakdown of the hormone. The addition of excess unlabeled JH at 15 min displaced labeled hormone from low-affinity sites and saturated the degradative enzymes. The net result of adding unlabeled JH would be to decrease the radiolabel in those fractions

TABLE 3. SUBCELLULAR DISTRIBUTION OF [^{3}H]-JH AFTER INCUBATION OF HOMOGENIZED LARVAL EPIDERMIS FOR 30 MIN AT 4°C WITH AND WITHOUT THE ADDITION OF EXCESS UNLABELED JH AT 15 MIN.

	% [^{3}H]-JH bound/50 μg protein per 30 min	
Fraction[a]	No addition[b]	Unlabeled JH added[c]
Nuclear	0.6	2.3
Mitochondrial	3.2	9.9
Membrane vesicles	71.0	56.0
Microsomes	4.1	10.9
Supernatant[d]	21.1	20.7

[a] Particulate fractions (F_1-F_6) were obtained after discontinuous sucrose gradient centrifugation of the 20,000 X g pellet.

[b] Homogenized epidermis was incubated with [^{3}H]-JH (3.36 X 10^{-8} M) for 30 min at 4°C.

[c] Homogenized epidermis was incubated with [^{3}H]-JH (3.35 X 10^{-8} M) for 15 min at 4°C then with unlabeled JH (3.36 X 10^{-4} M) for an additional 15 min at the same temperature.

[d] Free JH was not separated from bound hormone.

having low-affinity sites and increase the radiolabel in those fractions having high-affinity sites. On this basis, we have tentatively concluded that the nuclear, mitochondrial, and microsomal fractions contain high-affinity binding sites. However, these experiments are certainly not conclusive and we are currently investigating this aspect further.

Influence of RNase and DNase on [^{3}H]-JH Binding

Epidermal homogenates were treated with RNase and DNase prior to incubation with [^{3}H]-JH to determine if intact nucleic acids were involved in the binding of hormone. RNase and DNase catalyze the fragmentation of RNA and DNA (to nucleotides, nucleosides, and

and possibly nucleoprotein particles in the case of DNase) (Mahler and Cordes, 1966; Oosterhof et al., 1975). The effect of these treatments on the distribution of protein and isotope in subcellular fractions of epidermis are summarized in Fig. 3. Neither enzyme was effective in converting nuclear material into small soluble particles as evidenced by the lack of increase in protein and radioactivity in the microsomal + supernatant fraction. However, both enzymes fragmented nuclear material (as shown by the decreased protein in the nuclear fraction) into particles that sedimented primarily with the mitochondrial fraction and to a lesser extent with the membrane vesicle fraction. Fragmentation of material in the mitochondrial fraction by RNase is possible but could not be discerned because of the particles formed by the fragmentation of material in the nuclear fraction.

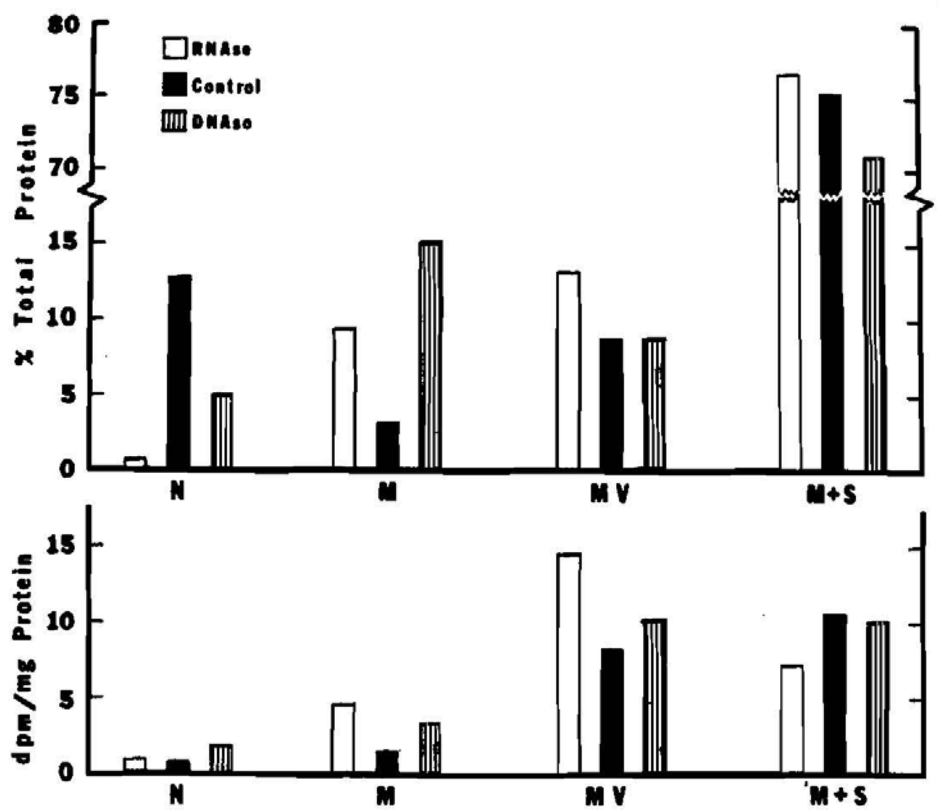

Fig. 3. Effect of incubation with RNase and DNase on [^{3}H]-JH binding in epidermal homogenates. Homogenized epidermis (0.09 g wet wt/ml) treated with RNase and DNase (see Materials and Methods) was incubated with 2.8 X 10^{-8} M [^{3}H]-JH for 30 min at 4°C. N, nuclear; M, mitochondrial; MV, membrane vesicles, and M+S, microsomal + supernatant. Values for dpm/mg protein were calculated on a relative basis.

The nuclear fragments which resulted from RNase incubation and which sedimented with the mitochondrial and membrane vesicle fractions were extremely effective in binding [^{3}H]-JH as shown by the increase in radioactivity/mg protein (calculated on a relative basis) (Fig. 3). On the other hand, DNase did not have such a pronounced effect and, in fact, the loss of "DNase fragments" increased the [^{3}H]-JH/mg protein in the remaining nuclear fraction. We conclude that DNase has some effect on the JH binding sites in the epidermal tissue, but that RNase has a more profound effect on both the size of the particle containing the binding sites and on the availability of the sites to bind JH.

DISCUSSION

Evidence has been presented that the carrier protein in *Manduca sexta* (Sanburg *et al*., 1975a,b) and *P. interpunctella** functions in the transport and protection of JH from degradation by enzymes in the hemolymph. The protection of JH by the carrier protein has been extended to other tissues in *Manduca sexta* (Hammock *et al*., 1975). Our data reported here indicate that epidermal target tissue contains similar enzymes which degrade JH and that the carrier protein influences such catabolism *in vitro*. However, we do not know whether such protection also occurs *in vivo* because it has not been demonstrated that the carrier protein-JH complex penetrates the target cell membrane.

JH was degraded most rapidly in the microsomal + supernatant, and membrane vesicle fractions and slowest in the mitochondrial and nuclear fractions. Addition of CPF largely protected JH from degradation in the cytosol, microsomal, and membrane vesicle fractions but not in the mitochondrial and nuclear fractions. In fact, more hormone was catabolized in the presence of the CPF in the latter two fractions. Our interpretation of these observations is that the fractions contained high-affinity binding sites for JH (possibly receptors) which selectively removed JH from the protective influence of carrier protein. This permitted the hormone access to degradative enzymes in the presence of the carrier protein. On the other hand, the microsomal plus cytosol fractions may not have contained high-affinity binding sites which allowed the carrier protein to exert its protective influence. The BSA binding experiment which demonstrated that general protein binding did not protect JH supports the speculation that high-affinity sites could remove JH from carrier protein but not protect the hormone. However, the possibility that the nuclear and mitochondrial fractions contained enzymes comparable to the carrier-bound-JH esterases in the hemolymph of *Manduca sexta* (Sanburg *et al*., 1975) has not been ruled out.

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The final step in the processing of JH is the transfer of the hormone from carrier protein to a receptor in the target tissue. This could be achieved by receptor recognition of the carrier protein-JH complex or of JH alone. However, the similar binding patterns in epidermal homogenates with JH alone or with the carrier protein-JH complex indicate that the carrier protein does not participate in subcellular recognition of JH.

Although the carrier protein-JH complex may not be required for subcellular recognition of JH, it may function by protecting the hormone until the high affinity receptors remove JH from the complex. Our binding data disclosed that nuclear, mitochondrial, and perhaps the microsomal fractions may contain such high-affinity binding sites. Furthermore, both isolated nuclei (Sekeris, 1972) and mitochondria (Firstenberg and Silhacek, 1973) have been shown to respond directly to JH by alterations in enzymatic activity. We therefore conclude that at least these two subcellular fractions should contain receptors for JH.

Although the chemical nature of JH receptors is not known, evidence is now accumulating that ribonucleoproteins are either receptors themselves or are closely associated with receptors for JH. This is supported by the work of Schmialek (1973) and by our observation that in the nuclear fractions RNase digestion and, to a lesser degree, DNase digestion caused formation of smaller nucleic acid fragments having a higher JH binding capacity. The specificity of these sites and their possible physiological significance remains to be determined. It is tempting to speculate that JH may bind in much the same way as steroids which bind nuclear and cytoplasmic ribonucleoprotein particles in target cells (Liang and Liao, 1974).

SUMMARY

A juvenile hormone (JH) carrier protein fraction (CPF) isolated from the hemolymph of *Plodia interpunctella* reduced binding but did not alter the relative binding patterns of JH in fractions of homogenized epidermis separated by differential and sucrose density gradient centrifugation. Bovine serum albumin (BSA) also reduced JH binding to the epidermal fractions. The CPF but not BSA protected the hormone from degradation by epidermal enzymes. Evidence is presented that suggests that high affinity binding sites for JH exist in the nuclear and mitochondrial fractions and that the JH carrier protein does not participate in recognition of the binding sites.

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REFERENCES

Anderson, J.N., Peck, E.J., and Clark, J.H., 1974, J. Steroid Biochem. 5:103.

Burdette, W.J., 1974, "Invertebrate Endocrinology and Hormonal Heterophylly," Springer-Verlag, New York.

Emmerich, H., and Hartmann, R., 1973, J. Insect Physiol. 19:1663.

Ferkovich, S.M., Rutter, R.R., and Anthony, D.W., 1974, J. Insect Physiol. 20:1943.

Ferkovich, S.M., Silhacek, D.L., and Rutter, R.R., 1975a, Insect Biochem. 5:141.

Ferkovich, S.M., and Rutter, R.R., 1975b, Wilhelm Roux' Arch., in press.

Firstenburg, D.E., and Silhacek, D.L., 1973, Experientia 29:1420.

Hammock, B., Nowock, J., Goodman, W., Stamoudis, V., and Gilbert, L.I., 1975, Molec. Cell. Endocrinol. 3:167.

King, R.J.B., and Mainwaring, W.I.P., 1974, "Steroid-Cell Interactions," University Park Press, Baltimore.

Kramer, K.J., Sanburg, L.L., Kézdy, F.J., and Law, J.H., 1974a, Proc. Nat. Acad. Sci. USA, 71(2):493.

Liang, T., and Liao, S., 1974, J. Biol. Chem., 249:4671.

Mahler, H.R., and Cordes, E.H., 1966, "Biological Chemistry," Harper and Row, New York.

Menn, J.J., and Beroza, M., 1972, "Insect Juvenils Hormones: Chemistry and Action," Academic Press, New York.

Oosterhof, D., Hozier, J.C., and Rill, R.L., 1975, Proc. Nat. Acad. Sci. USA, 72(2):633.

Sanburg, L.L., Kramer, K.J., Kézdy, F.J., and Law, J.H., 1975a, J. Insect Physiol. 21:873.

Sanburg, L.L., Kramer, K.J., Kézdy, F.J., Law, J.H., and Oberlander, H., 1975b, Nature, 253:266.

Schacterle, G., 1973, Anal. Biochem. 51:654.

Schmialek, P., 1973, Nature, 245:267.

Sekeris, C.E., 1972, Gen. Comp. Endocrinol. Suppl. 3:149.

Slade, M., and Zibitt, C.H., 1972, in "Insect Juvenile Hormones" (J.J. Menn and M. Beroza, eds.), pp. 155-176, Academic Press, New York.

Sláma, K., Romaňuk, M., and Šorm, F., 1974, "Insect Hormones and Bioanalogues," Springer-Verlag, New York.

Trautmann, K.H., 1972, Z. Naturforsch, 27b:263.

Whitmore, E., and Gilbert, L.I., 1972, J. Insect Physiol. 18:1153.